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16. ABSTRACT

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Analytical approaches to the determination of pesticides and metabolites in human and animal tissues will take many forms. Several factors must be considered in choosing an analytical scheme if the results are to be meaningful. Whenever possible the residue chemist will use standardized methodology which has been subjected to evaluation by collaborative studies. In the absence of such methods, the analyst must be aware of the capabilities and limitations of the various steps in the analytical procedure of choice. Extraction and cleanup efficiency must be considered in view of the determinative method to be used. Highly efficient gas chromatographic columns coupled with sensitive and selective detector systems are powerful analytical tools. One must be cautious, however, when relying on gas chromatographic peak retention time for compound identification. For a complete analysis, confirmatory techniques should be used. The available sample size may dictate the use of a micro method of analysis and, at the same time, preclude certain confirmatory techniques. Advantages of micro methods include, speed, smaller glassware and minimum solvent volumes.

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MICRO AND MACRO APPROACHES TO THE DETERMINATION OF PESTICIDE RESIDUES IN HUMAN AND ANIMAL TISSUES

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Summary

Analytical approaches to the determination of pesticides and metabolites in human and animal tissues will take many forms. Several factors must be considered in choosing an analytical scheme if the results are to be meaningful. Whenever possible the residue chemist will use standardized methodology which has been subjected to evaluation by collaborative studies. In the absence of such methods, the analyst must be aware of the capabilities and limitations of the various steps in the analytical procedure of choice. Extraction and cleanup efficiency must be considered in view of the determinative method to be used. Highly efficient gas chromatographic columns coupled with sensitive and selective detector systems are powerful analytical tools. One must be cautious, however, when relying on gas chromatographic peak retention time for compound identification. For a complete analysis, confirmatory techniques should be used. The available sample size may dictate the use of a micro method of analysis and, at the same time, preclude certain confirmatory techniques. Advantages of micro methods include: speed, smaller glassware and minimum solvent volumes.

Introduction

The determination of pesticide residues in human and animal tissues has been of concern to analytical chemists for many years. A wide variety of factors will influence the approach used in any particular analysis. Some history of the sample can be quite valuable. What compounds can be expected in the sample? Is it reasonable to look for other compounds? Should one analyze for the parent compound or is there a known metabolite that will be present instead? For example, dieldrin and heptachlor epoxide will be found in general population human adipose tissue as metabolites of aldrin and heptachlor. What are the expected levels of pesticides and/or metabolites? Is the selected methodology capable of the desired lower limit of detection with the size of sample available? Will the compounds of interest be quantitatively recovered with the analytical methodology used? What kind of confirmation is required? Is the sensitivity of the confirmatory technique commensurate with the sample size and/or cleanup procedures used? These are some of the questions that must be considered when approaching the determination of pesticides or metabolites in tissue samples.

The entire sample preparation procedure, be it macro or micro, must be considered when dealing with pesticide residue analysis. If losses of the compounds of interest occur at any step, the results of the analysis become questionable.

Extraction procedures for several grams of tissue generally call for blending with an organic solvent or grinding in a mortar with anhydrous sodium sulfate. The tissue may be minced and ground with sodium sulfate prior to Soxhlet extraction. Milligram quantities of tissue can be conveniently macerated in a small glass tissue grinder with an organic solvent. The resulting homogenate can be centrifuged and the solvent decanted. This process requires a minimum of glassware and solvent transfer, thus reducing chances of loss and background contamination.

The cleanup required for sample extracts will vary from minimal to most elaborate depending on the sample coextractives, and the desired level of detectability, sensitivity and selectivity of final detection systems. Sample cleanup in some cases provides preliminary separation of pesticides into groups thus enhancing the reliability of correct identification. Solvent partitioning is helpful in separating lipids and waxes from nonpolar pesticides but some pesticides may be partially lost at this step.

Absorption column cleanup is widely used in conjunction with solvent partitioning. Absorbents most frequently used include Florisil, silica gel, alumina and charcoal. An endless variety of solvents and solvent mixtures are applicable for the particular cleanup and/or separation desired. Exclusion chromatography or gel permeation chromatography has recently gained in popularity. Thin-layer or channel-layer chromatography may be substituted for column chromatography. This technique can provide good cleanup and separation with an absolute minimum of solvent interference at the determinative step. Acid or base partitioning may be used for cleanup of a limited number of the more stable pesticides and polychlorinated biphenyls (PCB's).

Depending upon the determinative step to be used, derivatization may be required to render the compounds of interest suitable for analysis. For gas chromatography, polar compounds may be converted to more volatile esters, ethers or amides prior to analysis. Derivatization may also be used to introduce heteroatoms into molecules thus making them responsive to element specific detectors. Fluorescent derivatives can be prepared which will be detected in the presence of large amounts of non-fluorescent background materials.

The final determination of pesticides extracted from tissues may take many forms. The most useful technique used today is probably gas chromatography (GC) interfaced with highly sensitive and selective detector systems such as electron capture, flame photometric and electrolytic conductivity. Alkali flame, microcoulometric and flame ionization detectors are also used. Several widely used GC columns are available which have been evaluated extensively. The literature abounds with relative retention data for hundreds of pesticides on these columns under vigorously standardized conditions. In many instances, tentative identification of unknowns may be made on the basis of relative retention alone. This does not suggest that the analyst should rely on only relative retention data, but that his choice of pesticide standards to try can be greatly narrowed.

Thin-layer chromatography (TLC) finds use in residue analysis for quantitation and confirmation of results obtained by other means. Recent developments in scanning techniques show promise for improving the quantitative aspects of TLC.

Colorimetric and paper chromatographic techniques are still in use, but suffer from lack of sensitivity and specificity.

Confirmatory techniques for suspected pesticides or metabolites in residues range from the quick and simple p-value to the most elaborate and sophisticated instrumental analyses. In laboratories where expensive equipment is not available, there are alternate means for confirmation of residues that are of reasonable reliability. One such means is chemical derivatization followed by gas chromatography or other suitable qualitative techniques. Where sufficient residue is present, TLC can be used effectively. p-values yield limited information, but in the absence of other confirmatory means, some confidence can be gained. In addition, p-values can be obtained on as little as 100 microliters of sample extract.

Confirmation by instrumental methods has proved to be quite valuable. Infrared spectra matching provides very good evidence where sufficient compound is present. Mass spectra, especially when obtained in conjunction with gas chromatography, is an excellent means of confirmation.

Macro Methods

Historically, pesticide residues were determined by colorimetric techniques after extraction and occasional cleanup. Paper and thin-layer chromatographic methods were, and still are, used for multiple residues. These methods, though suitable for many applications, suffer from lack of sensitivity and specificity. Fairly large sample sizes are usually required and rigorous cleanup is needed. With the advent of gas chromatography and the associated selective and sensitive detector systems, a wider range of analyses and lower limits of detection of pesticide residues became possible. Many of the extraction and cleanup procedures were directly applicable to the new GC methods.

The multiresidue procedure outlined in the FDA Pesticide Analytical Manual[1] is probably one of the most widely used methods for pesticide residues. This procedure is based on the original work by Mills[2], and Mills et al[3], and has evolved over the years to include a sizeable number of pesticides and metabolites in a multitude of sample types. The essentials of extraction with an organic solvent, partitioning between two immiscible solvents and column chromatography on Florisil remain an effective means of sample preparation. Modifications of this methodology have been applied to almost every conceivable substrate. In general, this methodology has been used on a macro scale (greater than one gram of sample) but micro modifications have been reported. Alternate elution solvents have been used to improve upon the lipid/pesticide separations.[4]

The Environmental Toxicology Division of EPA has adapted the Mills method with minor modifications and included it in their Manual of Analytical Methods[5]. This modification is used in the Human Tissue Monitoring Program for the determination of pesticide residues in adipose tissue. The methodology has been subjected to collaborative study, and in conjunction with standard GC columns and instrumented parameters, has produced very acceptable interlaboratory precision and accuracy.

Also included in the EPA Manual is a micro modification which can be used for tissue samples of a few hundred milligrams.[6] In many instances pesticide determinations are required for biopsy samples or for small laboratory animals where most of the sample has been used for other determinations.

Some success has been reported with a steam codistillation procedure[7] for the cleanup of oils and fats. The sample extract in an organic solvent is injected into a heated tube and the pesticides are flushed through with additional solvent leaving the coextracted interferences behind. A micro Florisil column is required for the determination of chlorinated hydrocarbons. It was reported that sample preparation time could be reduced from 2-4 hours to 30-50 minutes. Up to one gram of oil or fat could be cleaned up.

Gel permeation chromatography has been used as a cleanup technique for the determination of chlorinated hydrocarbon pesticides in fish tissue.[8] This system was subsequently automated[9] and evaluated.[10] Twenty-three sample extracts can be loaded into the commercially produced unit and the cleanup is subsequently accomplished unattended. Due to the carryover of lipid into the pesticide fraction 1.1ppm of chlorinated hydrocarbon in one gram of fats and oils is considered a practical lower limit without additional cleanup. With this system the potential exists for various column packings and eluting solvents. As such, the cleanup would probably be considered to be on the macro scale. Use of other adsorbents could most likely allow handling of micro samples. Presently the large column bed of gel (450gm) will handle up to about one gram of lipid. Simply scaling down the size of the cleanup column would allow as little as 50 to 100mg of sample to be cleaned up but the minimum practical level of determination would still be 0.1ppm. Combined with other cleanup techniques, this system has been applied to the separation of ultra trace quantities of dioxin from lipids.[11]

PCB's were first recognized as environmental contaminants in the mid 1960's. Since that time methodology has been developed to chromatographically separate PCB's from pesticides thus allowing more confident identification.[12,13] Variations of this procedure have been used in conjunction with many different multiresidue procedures. Confirmatory techniques such as GC-MS and chemical derivatization have proved to be valuable.

Micro Methods

When the residue chemist is confronted with very small samples such as tissue biopsies the analytical approach must be quite different than that used when unlimited sample is available. In such instances the entire sample may be required, thus precluding the possibility of a "rerun". Reliable and accurate methodology is a prerequisite. One must consider that 100mg of sample containing a few nanograms of pesticide or metabolite can easily get lost on the surfaces of large glassware and column adsorbents. Concentrating several hundred ml of eluting solvent to a very small volume so that the desired amount of pesticide can be introduced into the analytical system can obviously create interference problems.

Numerous micro methods have been developed for application to the cleanup and analysis of tissue for pesticide residues. Adsorbents used for columns range the entire gamut as do elution solvents. In many cases the investigator is attempting to clean up and separate multiple pesticide residues in one step. Adsorption column chromatography is a valuable asset for compounds which are difficult to separate by GC. In addition,

selective elution of pesticides adds confidence to the identification of suspected pesticides.

Kadoum[14,15,16,17] devised separation schemes for several chlorinated hydrocarbon and organophosphate pesticides on silica gel. In this series of papers he investigated various solvent systems in attempts to clean up plant, animal, soil and water extracts. As with most micro methods, more samples could be handled in a shorter period of time and solvent volumes were kept to a minimum.

Leoni[18] used a silica gel system similar to that reported by Kadoum, and demonstrated the cleanup and separation of fifty pesticides into four groups. His interest was primarily in the analysis of water.

Holden and Marsden[19] used alumina and silica gel columns in sequence to clean up animal tissues for residue determinations. The alumina was more effective in removing lipid from the extract while silica gel was useful for separating the pesticides into groups. One hundred milligrams of tissue equivalent was chromatographed, thus lending this technique to micro analysis.

Law and Goerlitz[20] evaluated micro columns of alumina, silica gel and Florisil for the cleanup and group separation of pesticides in water. The micro Florisil column was prepared in a disposable pipet and the eluting solvents used were 6% and 15% ethyl ether in hexane. This system is essentially a scaled down Mills[2] Florisil column. These authors noted several advantages of micro columns including speed, convenience and recovery.

Johnson[21] used a one gram column of silica gel, deactivated with 1% water, to clean up water sample extracts. Chlorinated pesticides were separated into two fractions before quantitation by electron capture GC.

Recently, Erney[22] reported on the use of miniature Florisil columns for the separation and cleanup of chlorinated pesticides. The column and eluting solvents were the same as those used in the official FDA procedure but the size was reduced by about one fifth. Separations and recoveries were found to be comparable to that obtained with the larger column.

Erney[23] also described a miniature silica gel column that was used for the separation of some PCB's, DDT and analogs. Five grams of silica gel was slurry packed in a 1cm i.d. column and eluted with hexane and methylene chloride.

A micro silica gel column separation scheme was developed by Sherma and Shafik[24] and used in conjunction with air sampling for pesticides. Group separation was obtained by collecting three fractions of 15ml or less from a 1gm silica gel column. Chlorinated hydrocarbon, organophosphate and carbamate pesticides were quantitated. The carbamates were derivatized with pentafluoropropionic anhydride prior to electron capture gas chromatography. Adaptation of this methodology for the determination of residues in other sample types should be possible.

Enos et al[6] developed a micro method for the determination of pesticide residues in several different tissues. Samples of 500mg or less are extracted with acetonitrile, diluted with aqueous salt solution and partitioned against hexane. The hexane layer is concentrated to a small volume and transfered to a column of 1.6gm of Florisil. The column is eluted successively with hexane followed by 1% methanol in hexane. The two fractions collected are concentrated to a suitable volume and analyzed by gas chromatography on standard columns using an electron capture or flame photometric detector. A modification of this methodology for pesticides in human milk is included in the EPA Analytical Manual[9].

Applications

The recent interest in hexachlorobenzene (HCB) prompted us to look for ways to improve the recovery of this compound from adipose tissue.[25,26] Both the Mills procedure and the micro modification as outlined in the EPA Analytical Manual yielded about 60% recovery. With the Mills modification, losses of HCB occurred at the acetonitrile/petroleum ether partitioning step. The low recovery with the micro procedure was traced to the acetonitrile extraction step.

The recovery of HCB in the micro method was improved to 80% or better by simply changing the extraction solvent to 2% acetone in acetonitrile. Recovery of other commonly found chlorinated pesticides was not altered.

Attempts to improve the recovery of HCB with the Mills procedure were not successful. However, a simple and quick method was worked out which yielded close to 100% recovery. Fat samples of 500mg or less were extracted with hexane and transfered directly to a standard 100 g 3% Florisil column. Hexane (100ml) was used as the eluting solvent and yielded quantitative recovery at HCB levels as low as 5ppb with a minimum of interference. Incomplete recovery of most other chlorinated pesticides was noted, and as a result, this procedure could not be used as a multiresidue method. This technique would, however, be useful in conjunction with the Mills method by reserving 0.5gm equivalent of the original sample extract and carrying the major portion (2.5-5gm) through the standard multiresidue routine. If HCB were suspected in the sample, the "direct elution" method could be used for the remaining extract for more reliable quantitation of HCB.

A rapid and simple technique for the confirmation of HCB in adipose tissue was also developed. Mono- and disubstituted ether derivatives of HCB were formed by reaction with KOH in various alcohols. Quantitative and qualitative data for HCB derivatives were obtained on standard GC columns coupled with an electron capture detector.

During the course of a rat feeding study, the need arose to determine residues of the fungicide dichloran (2,6-dichloro-4-nitroaniline) in various tissues and excreta.[27] A micro procedure was required because of limited sizes of some samples such as eye tissues. Only slight modification of the micro procedure of Enos et al[6] was required to obtain acceptable recovery. Because of the polar nature of the compound, it was necessary to reduce the volume of the acetonitrile extracting solvent before partitioning the dichloran into hexane. It was also found that a larger volume of 1% methanol in hexane was required for the second fraction from the Florisil column. Several standard GC columns were found to be acceptable for quantitation with an electron capture detector. The average overall recovery of the method for eight tissues and excreta was greater than 90%. Liver and brain were the most troublesome tissues and yield recoveries of 82 and 84%, respectively.

When the residue chemist is faced with the task of quantitative determination of a particular compound or group of compounds, he can many times use existing methodology even though the procedures have not been applied to his specific need.

Advantages and Disadvantages

Micro methods have several advantages which in many cases are overlooked. Analysis time can be substantially shorter with a small cleanup column. Consider the time required for elution of several hundred ml of solvent through a large column and add to that the time for concentration to a suitable volume for the determinative step. Elution through a micro Florisil column can be accomplished in about one fifth the time. Large volumes of solvents can be awkward to handle and expensive in the long run. Smaller pieces of glassware are more easily handled and provide less surface area on which to lose pesticides.

Micro cleanup columns can be time savers when one is screening various adsorbents and solvents in search of suitable elution patterns. Many columns can be run in a short period of time with minimum solvent volumes. Once an acceptable system has been found, the column size can easily be scaled up if desired.

When only very small samples are available the use of a micro procedure is mandatory. Attempts to work up 100mg of a tissue with methodology designed for 10 to 100gm are certain to fail, if for no other reason than the tremendous reagent blank that would surely arise.

If unlimited sample is available, but the chemist chooses to use a micro analytical method, he must concern himself with obtaining a representative subsample that will reflect the true level of pesticide or metabolite in the total sample. Use of a large sample for extraction and taking an aliquot from the total extract for cleanup by a suitable micro method would produce such a representative sample. Micro methods of analysis limit the range of confirmatory techniques that can be used. TLC, ultraviolet, infrared and some mass spectrometric techniques are not sensitive enough to detect suspected residues in extracts from small scale sample preparations. Derivatization and p-value procedures can provide valid confirmatory information for nanogram quantities of pesticides or metabolites.

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